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# Folding of outer membrane protein A in the anionic biosurfactant rhamnolipid

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ABSTRACT

**Folding and stability of bacterial outer membrane proteins (OMPs) are typically studied in vitro using model systems such as phospholipid vesicles or surfactant. OMP folding requires surfactant concentrations above the critical micelle concentration (cmc) and usually only occurs in neutral or zwitterionic surfactants, but not in anionic or cationic surfactants. Various Gram-negative bacteria produce the anionic biosurfactant rhamnolipid. Here we show that the OMP OmpA can be folded in rhamnolipid at concentrations above the cmc, though the thermal stability is reduced compared to the non-ionic surfactant dodecyl maltoside. We discuss implications for possible interactions between OMPs and biosurfactants *in vivo*.**



## 1. Introduction

Outer membrane proteins (OMPs) reside in the outer membrane of gram-negative bacteria and also in chloroplasts and mitochondria, reflecting the bacterial origin of these organelles. Outer membrane proteins have also been identified in secreted outer membrane vesicles [1,2] and 30% of the proteins in the extracellular matrix (biofilm) of *Pseudomonas aeruginosa* have been identified as OMPs [3].

OMPs carry out different tasks in the membrane. They work as protein translocators and folding catalysts for other OMPs [4], adhesins for bacterial infection [5], passive diffusion pores and efflux channels [6], siderophore receptors [7] and enzymes [8–11], e.g. lipases, proteases and palmitoyl transferases. The OMP fold consists of 8–24 β-strands oriented in an antiparallel manner to form a β-barrel. In addition to the membrane-embedded β-barrel, OMPs may also have extramembraneous domains that protrude either into the periplasm or out into the extracellular space.

Folding and stability of several OMPs have been studied in surfactants as well as in phospholipid vesicles [12–15]. Each system provides distinct advantages and disadvantages for folding of OMPs [16]. Surfactants are usually highly soluble and micelles are easily compatible with spectroscopic assays, making them very convenient tools to study OMPs. The lipid bilayer is often perceived as a more native-like environment as opposed to the more artificial environment of a synthetically produced surfactant micelle. However, while vesicles composed of phospholipids closely resemble lipids of the native inner membrane, the outer membrane of gram-negative bacteria is more complex, with an inner leaflet of phospholipids and an outer leaflet composed of lipopolysaccharides (LPS). Thus, although OMPs are stabilized in phospholipid vesicles [17], these vesicles do not represent their native environment. However, vesicles composed of an asymmetric bilayer are difficult to prepare and are typically only stable on a short timescale [18]. Folding in surfactant requires micelles, i.e. surfactant concentrations above the critical concentration (cmc) and OMPs generally only fold in neutral or zwitterionic surfactant micelles [19]. Thus OMPs can only be folded in SDS micelles if co-formulated with a large excess of neutral surfactants [20] or amphipathic mono- and di- alcohol osmolytes [21]. The only exception to this rule is provided by anionic LPS which when isolated can fold OMPs [22].

Surfactants need not only be of synthetic origin. Several microorganisms produce so-called biosurfactants [23] to exploit their

*Abbreviations:* BS, biosurfactant; DDM, dodecyl maltoside; OMP, outer membrane protein; RL, rhamnolipid; SDS, sodium dodecyl sulphate; LPS, lipopolysaccharide

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surface active properties. Biosurfactants (BS) are very diverse in structure. Glycolipids constitute a major class of BS and are produced and exported to the extracellular environment by a number of different microorganisms. They have received increasing attention, mostly because of their potential as substitutes for synthetic surfactants in detergents, cosmetics, pharmaceuticals, etc. Glycolipid production yields of up to 400 g/L have been reported [24]. The gram-negative opportunistic pathogen *P. aeruginosa* produces the glycolipid biosurfactant rhamnolipid (RL), which consists of one or two rhamnose sugars attached to β-hydroxy-decanoic acid chains. Importantly, RLs contain a carboxylate with a $pK_\alpha$ between 4.3 and 5.5 (increasing with micelle formation) [25], making RL an anionic biosurfactant at neutral or alkaline pH. Rhamnolipid production by *P. Aeruginosa* in bioreactors reaches levels of almost 50 g/L [26]. Patents claim production concentrations of over 100 g/L [27]. Furthermore cystic fibrosis patients infected with *P. Aeruginosa* showed presence of rhamnolipid, with levels ranging from 8 μg/ml (sputum) [28] to 65 μg/ml (lung secretions) [29].

Proposed *in vivo* functions of RL include solubilization of hydrophobic substrates, modification of surfaces, anti-microbial properties, involvement in biofilm development [30] and more. RL has also been suggested to increase cell surface hydrophobicity by removing LPS from the outer membrane, [31], as well as increasing protein content in the extracellular environment [32]. Protein release peaks above RL cmc (~50–100 mg/L) but does not adversely affect bacterial growth [32]. These observations led us to the hypothesis that interactions between anionic rhamnolipid biosurfactants and OMPs may have physiological relevance. Here, we show that RL micelles provide an environment that supports folding of outer membrane proteins. This is to our knowledge the first report of an anionic surfactant (apart from the lipid-like LPS) that supports folding of OMPs.

## 2. Materials and methods

### 2.1. Materials

Glycine, EDTA, Phenylmethylsulfonyl fluoride (PMSF) and Trypsin were from Sigma (St. Louis, MO). Dodecyl maltoside (DDM) was from Anatrace (Maumee, OH). JBR515 rhamnolipid (RL) was provided by Jeneil Biosurfactant Company (Saukville, WI, USA) as a liquid solution consisting of 15% RL of highest grade. JBR515 is a 0.35:1 mixture of mono-RL and di-RL with molecular weights of 504 and 650 Da, respectively. The transmembrane domain of the OmpA gene, TM-OmpA (a construct containing residues 1–176 of the N-terminal domain without the preceding leader sequence, followed by the C-terminal sequence Arg-Ser-$(His)_6$) was cloned, expressed and purified as described [33]. The TM-OmpA stock used in the experiments described below was 19 mg/ml (978 μM) in 8 M of urea.

### 2.2. Determination of the critical micelle concentration by pyrene fluorescence

The cmc of RL was determined by pyrene fluorescence as described in [34]. Briefly, different concentrations of RL in buffer A (10 mM Glycine pH 10 and 2 mM EDTA) were prepared. After equilibration for 30 min, pyrene was added from a 100 μM stock in ethanol to a final concentration of 1 μM. Fluorescence scans were performed on a LS-55 luminescence spectrometer (Perkin–Elmer Instruments, UK), using an excitation wavelength of 335 nm, emission from 360 to 410 nm and excitation/emission slits of 5/3.5 nm. The ratio of the emission peaks at 372.5 ($I_1$) and 383.5 nm ($I_3$) is used to evaluate the polarity of pyrene's environment and thus determine the cmc [35].

### 2.3. Folding verification by SDS–PAGE band shift assay

TM-OmpA folding in RL at concentration below and above the cmc was monitored via SDS–PAGE. TM-OmpA was diluted to a final concentration of 0.2 mg/mL into the refolding buffer containing buffer A and RL from 0–2.56 mM. Samples were incubated for two days at room temperature and 20 μL of each sample was mixed with 6× sample buffer, after which 9 μL was analyzed by SDS–PAGE without prior boiling.

### 2.4. Determination of secondary structure and thermal stability by circular dichroism

TM-OmpA was diluted to a final concentration of 0.2 mg/mL (~10 μM) in the presence of 1 mM of RL or DDM in buffer A and incubated overnight at 37 °C. Far-UV CD spectra were recorded, using a 1.0-mm quartz cuvette on a JASCO J-810 spectropolarimeter (Jasco Spectroscopic Co. Ltd., Japan) equipped with a Jasco PTC-423S temperature control unit. Wavelength scans were recorded in the wavelength range of 200–250 nm, with a bandwidth of 2 nm and a scanning speed of 50 nm/min. Six accumulations were averaged and buffer background contributions subtracted. Thermal scans were performed on 0.1 mg/mL TM-OmpA pre-incubated with 1 mM of RL or DDM in buffer overnight, scanning 20–95 °C at 1 °C/min, monitoring at 205 nm and fitting data to a two-state thermal transition [36]. The TM-OmpA sample for thermal scan in SDS was prepared by first refolding 0.2 mg/mL TM-OmpA in 2 mM RL in buffer A overnight at 37 °C. The sample was then mixed 1:1 with 40 mM SDS in buffer A. CD thermal scans were performed as above.

### 2.5. Trypsin proteolysis

0.4 mg/mL TM-OmpA was incubated with buffer A or with 10 mM of surfactant in buffer A for 2 days at 37 °C. From a 1 mg/mL stock in 1 mM HCl pH 3, trypsin was added to 0.04 mg/mL. After 2 h at 37 °C, 100 mM freshly prepared PMSF in isopropanol was added to 2 mM. Protein samples were mixed 1:5 with 6× loading buffer without DTT. 3 μL was analyzed by SDS–PAGE analysis without boiling. All SDS–PAGE experiments were carried out using a 15% polyacrylamide separation gel [37].

### 2.6. Folding kinetics of TM-OmpA

Folding kinetics of TM-OmpA in RL and DDM was monitored on a Cary Eclipse fluorimeter (Varian) and a 10 mm quartz cuvette with magnet stirring. Excitation and emission wavelengths were 280 and 330 nm respectively, and slit widths were 5 nm. Refolding experiments were conducted by mixing buffer and the desired concentration of surfactant in the cuvette. Under magnet stirring, concentrated and unfolded OmpA was then added to a final concentration of 2 μM and fluorescence was followed over time. Data were fitted to a single exponential decay in Kaleidagraph 4.0 (Synergy Software).

## 3. Results

### 3.1. TM-OmpA is able to refold in rhamnolipid

To determine if RL could support folding of OMPs, we incubated the well-studied [14,15] transmembrane domain of OmpA (TM-OmpA) with 0–3 mM RL. After incubation with RL overnight at 37 °C, TM-OmpA folding was analyzed using the SDS–PAGE band-shift assay (Fig. 1A). Folded TM-OmpA resists unfolding in SDS (unless boiled) and migrates more rapidly on SDS–PAGE than

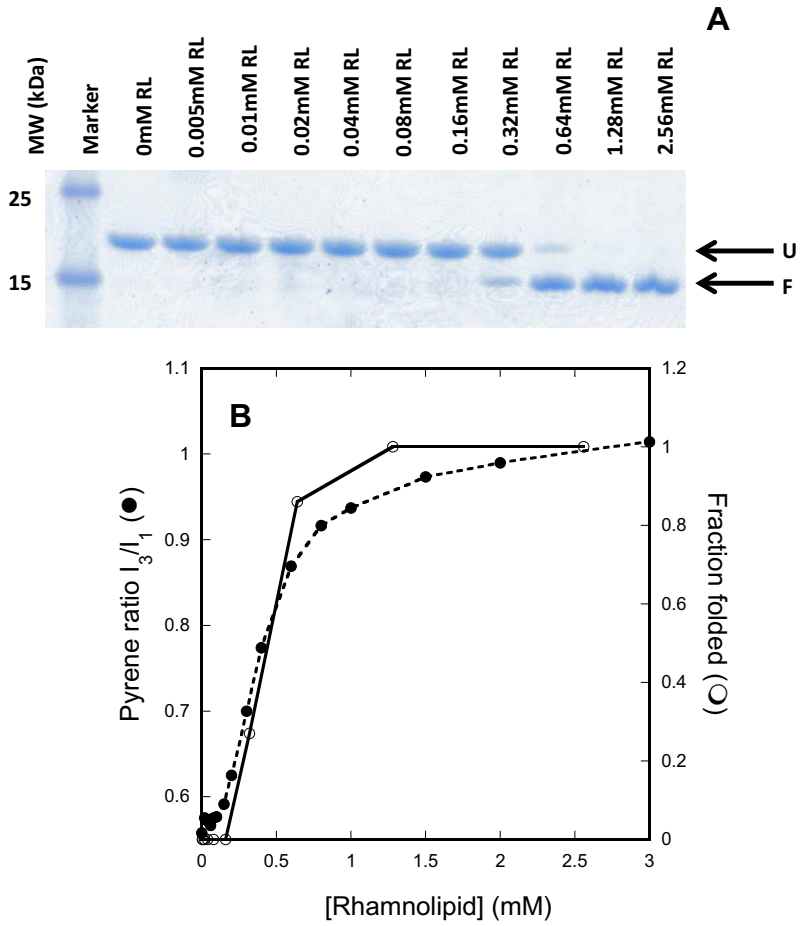


**Fig. 1.** Folding of TM-OmpA in RL at concentrations below and above the cmc. (A) Folding tested by SDS–PAGE band shift assay. Band shift occurs around 0.32 mM RL indicating folding. (B) Pyrene $I_3/I_1$ ratio changes from 0.58 to 1 between 0.2 and 1 mM RL showing the concentration range where RL micelle are formed. (B) The fraction of folded TM-OmpA based on densitomeric analysis of data in panel A correlates closely with pyrene fluorescence, showing that TM-OmpA only folds in the presence of RL micelles.

unfolded TM-OmpA because of its more compact conformation. A clear band shift appeared only >0.16 mM RL (Fig. 1A). Band shift was incomplete at 0.32 mM RL where unfolded TM-OmpA dominated. At 0.64 mM the folded band dominates and at higher RL concentrations only folded TM-OmpA was observed.

To determine if the band-shift was correlated to the cmc of RL, we determined RL's cmc under the applied buffer conditions by pyrene fluorescence. The ratio between the emission peaks $I_3$ and $I_1$ was ~0.58 in the absence of RL and increased to 0.95 between 0.2 and 1 mM RL, after which little increase was observed (Fig. 1B). Thus micelles will be present $\geqslant$0.2 mM RL. The fraction of folded OmpA, determined by densitometry of the bands in Fig. 1A, closely follows the change in pyrene fluorescence (Fig. 1B). This strongly indicates that TM-OmpA folding requires RL micelles.

To obtain independent confirmation that OmpA was folded correctly in RL micelles, a proteolysis assay was performed. Trypsin will degrade unfolded OmpA while folded OmpA will be protected. SDS–PAGE showed that OmpA incubated with RL or DDM micelles was protected from trypsin proteolysis, while OmpA incubated only with buffer was completely digested (Fig. 2).

To complement these assays, we investigated the secondary structure of OmpA by far-UV CD. The spectra of OmpA in buffer and in RL are very different. In buffer, the spectrum indicates a large proportion of unordered structure, while the minimum at 214 nm for OmpA in RL indicates a large proportion of β-sheet structure (Fig. 3A). This spectrum is essentially identical to that

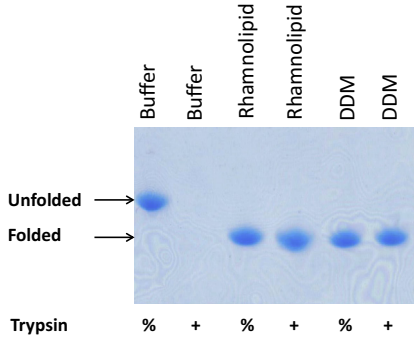


**Fig. 2.** Folding of TM-OmpA probed by trypsin digestion. TM-OmpA was folded in 10 mM RL or DDM at 37 °C overnight and subsequently digested with trypsin. Unfolded TM-OmpA in buffer was readily degraded by trypsin, whereas TM-OmpA folded in both DDM and RL resisted trypsin attack.

of OmpA folded in DDM, showing that OmpA attains the same degree of β-sheet structure in RL as in DDM.

In summary, these results provide clear evidence that TM-OmpA can be folded in RL micelles.

### 3.2. Thermostabiliy of TM-OmpA

Although RL is able to promote folding of OmpA, it was of interest to elucidate whether OmpA is stabilized to the same extent in

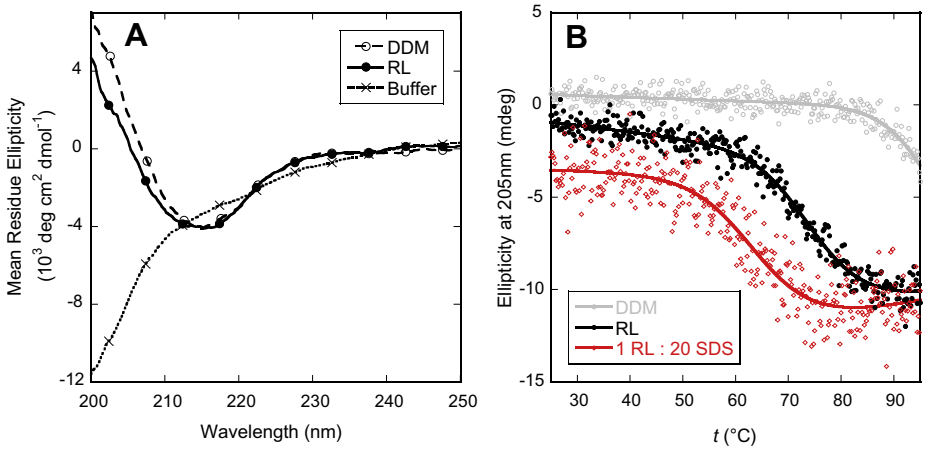


**Fig. 3.** Secondary structure and thermal stability of TM-OmpA in DDM and RL monitored by circular dichroism. (A) Far-UV spectra of TM-OmpA in DDM and RL are almost identical and indicate a high degree of β-secondary structure. In contrast a large degree of random coil is observed for TM-OmpA in buffer. (B) Thermal stability followed at 205 nm. TM-OmpA folded in RL shows a melting midpoint of 74.7 ± 1.3 °C while TM-OmpA folded in DDM is only partially unfolded at 95 °C. Refolded TM-OmpA to which SDS in excess has been added show a melting midpoint of 64.4 ± 1.6 °C.

RL compared to non-ionic surfactants. Thermal scans of OmpA in RL and DDM using far-UV CD revealed significant differences between the two surfactants. TM-OmpA folded in DDM only started on its melting transition around 90 °C (Fig. 3B), while TM-OmpA folded in RL melted with a midpoint transition of 74.7 ± 1.3 °C. This indicates that while TM-OmpA can fold in RL, the thermal stability is significantly lower than for DDM. For comparison, we also investigated the thermostability of OmpA in SDS. After OmpA had been refolded in RL overnight at 37 °C, SDS was added in 20 M excess and a CD thermal scan was performed. The midpoint transition of the melting curve was 64.4 ± 1.6 °C. This shows that while OmpA is thermally destabilized in RL when compared to DDM, it is significantly more stable in RL than in SDS.

### 3.3. Folding kinetics in RL

We investigated by fluorescence how quickly OmpA refolds in the presence of 1–10 mM RL (Fig. 4A), compared to folding in the presence of 1–10 mM DDM (Fig. 4B). At all concentrations, data were satisfactory fitted using a mono-exponential decay and there was a clear linear correlation between the rate constant from these fits and surfactant concentration (Fig. 4C). Refolding rates are slightly faster in DDM than in RL, ranging from >4-fold faster at 1 mM to <2-fold faster at 10 mM. To probe the reaction in more detail, we plotted the initial rate of folding (i.e. the slope of the curves in Fig. 4A and B at time $t = 0$) against surfactant concentration in a double-logarithmic plot. For RL, this gives a linear fit with a slope of 1 (Fig. 4D), indicating that folding is a first-order reaction with respect to RL concentration [38]). Interestingly, a similar analysis with DDM using data from Fig. 4B gives rise to a linear fit with a slope around 0.6 (Fig. 4D), suggesting that DDM micelles do not participate to the same extent as RL in protein interactions during the folding reaction.

## 4. Discussion

We show that the outer membrane protein OmpA can be folded and stabilized in the anionic biosurfactant rhamnolipid at concentrations above RL's cmc. Correct folding was verified by combining SDS–PAGE, trypsin proteolysis and CD spectroscopy. OmpA thermal stability is reduced in RL compared to the non-ionic surfactants DDM at 1 mM surfactant, where the protein folds ~10-fold more slowly in RL than in DDM micelles. It is not surprising that RL destabilizes OmpA compared to DDM. Anionic surfactants such as SDS destabilize proteins because they bind to proteins with much higher affinity than uncharged surfactants through electrostatic and hydrophobic interactions which are most accessible in the denatured state [39]. It is however remarkable that RL – unlike SDS – will support refolding (albeit with lower refolding kinetics than at the corresponding DDM concentrations). This indicates that RL have properties resembling both ionic and non-ionic surfactants, i.e. both stabilizing and destabilizing properties. RL is able to destabilize globular water-soluble proteins such as myoglobin and α-Lactalbumin, binding to these proteins in approximately the same protein: surfactant mass ratio as SDS (K.K.A., unpublished observations). Since RL is twice as large (R1 = 504 Da and R2 = 650 Da) as SDS (265 Da, without Sodium) and its carboxylate is a weaker acid than the sulfate group of SDS, RL binds at a lower molar ratio and will have a less deleterious effect on protein stability than SDS. The carboxylate likely enables RL to interact more strongly with OmpA than DDM does, and may also explain why the refolding kinetics increase more strongly with RL concentration than with DDM concentration. The reduced thermostability of OmpA in RL compared to DDM is not necessarily critical for interactions between RL and OMP *in vivo*, since OMPs are kinetically stable [40]. That is, once the native structure has formed, OmpA unfolds only very slowly even under denaturing conditions. This allows OMPs to maintain activity and functionality over long time periods even under harsh conditions.

While OMPs have not been shown to fold in pure anionic surfactants, they have been used with both non-ionic surfactants and alcohols in mixed micelle systems that support OMP folding. Thus, in the binary surfactant system of Octyl Glycoside (OG) and SDS, OmpA folds but only at OG mole fractions >0.79 [20]. OmpA can also be stabilized in mixed micelles of SDS and different alcohols [21], but folding requires a large excess of alcohol. In both cased the charge density of the micelles has to be decreased in order to provide a stabilizing folding environment. Negative carboxylate groups have also been introduced into amphipols which are surface active polymers designed for the stabilization of membrane proteins [41]. The most studied amphipol is A8–35 and approximately 1/3 of the monomers that constitute the polymer contain a free carboxylate group. The carboxylate groups are however separated by isopropylamide spacer groups that lower the

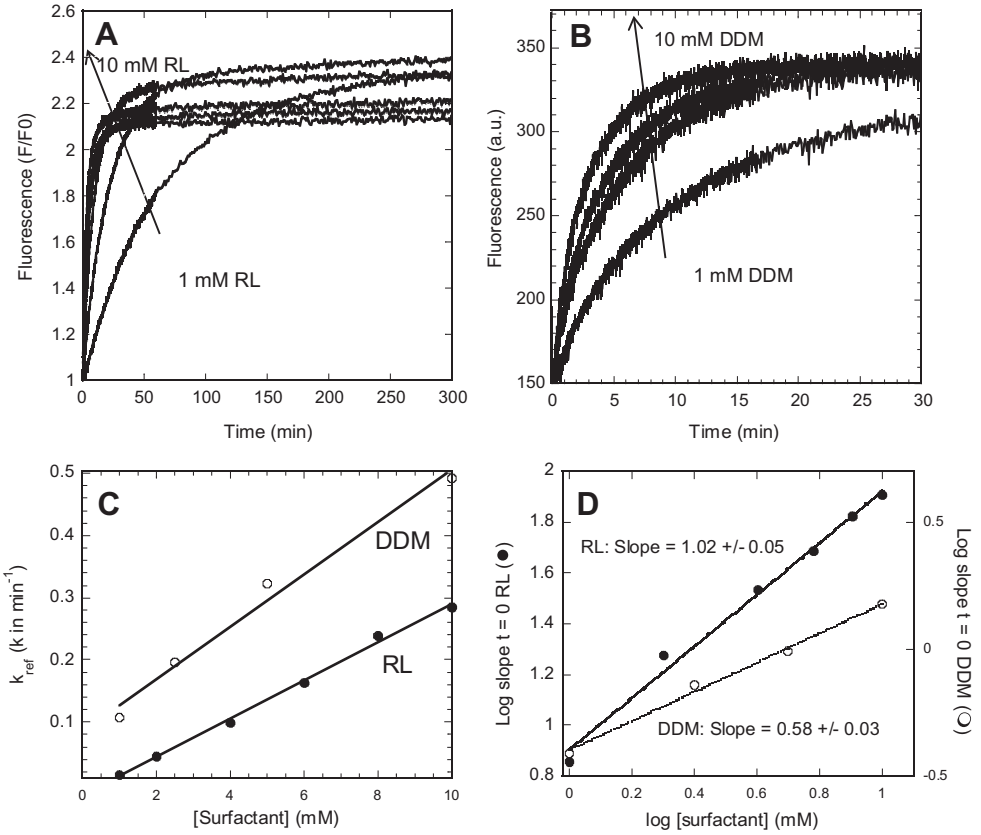


**Fig. 4.** Kinetics of TM-OmpA folding in RL and DDM. Raw data for refolding of TM-OmpA in (A) RL and (B) DDM. (C) Rate constants for refolding from panels A and B plotted as function of surfactant concentration with best linear fits indicated. (D) Initial slopes obtained from panels A and B are plotted versus surfactant concentration in a log–log plot and the slopes to the best linear fits are indicated. Note that although different *y*-axes are used, the axis range is the same on both *y*-axes (1.2 units), allowing direct comparison.

charge density of the polymer. Together this indicates that charge density and type of anionic group are factors that influence the stabilization of OMPs in macro-molecular structures such as micelles.

### *4.1. Biological implications: the role of RL in secreting OMPs*

The fact that RL can stabilize OMPs may have implications for our understanding of *in vivo* export mechanisms. RL can alter the outer membrane by extracting LPS from the outer membrane [31], and RL also leads to higher protein content in the extracellular space [32]. Together with our finding that RL are able to stabilize OMPs it is likely that RL not only extract LPS from the outer membrane but also OMPs. Since a number of OMPs are enzymes, RL mediated extraction from the outer membrane may transfer active enzymes and other proteins with functionality into the extracellular environment. Such a transfer is likely to be very general and will not target specific OMPs. While not a membrane protein, flagellin has been shown to form complexes with RL and this complex can cross the epidermis [42]. A recent study of the extracellular matrix (biofilm) of *P. aeruginosa* showed that 30% of proteins were OMPs [3]. *P. aeruginosa* production of RL is regulated by quorum-sensing system and RL production is not initiated until the late exponential/early stationary phase when the substrate and other important growth metabolites are scarce [43,44]. There are thus certain similarities to outer membrane vesicles (OMVs) which gram-negative bacteria use to transport bio-molecules including functionally active OMPs [45].

On a more applied note, small apolar molecules or proteins engineered to target the outer membrane could be exported extracelluarly in RL micelles in a soluble state despite high hydrophobicity. This approach could be a useful complement to emerging efforts to release water-soluble molecules, e.g. small interfering RNA, from bacteria to eukaryotic cells in secreted outer membrane vesicles [46]. Such micelles will most likely also contain lipid components from the outer membrane, including LPS. OMP and LPS co-extraction may be important because many OMP enzymes have LPS binding motifs and LPS is required for activity [9,47–49], just as LPS alone can fold and stabilize OMPs [22].

Although OmpA and RL derive from two different bacteria (*Escherichia coli* and *P. aeruginosa*, respectively), RL's ability to support OMP folding is probably a general phenomenon. SDS–PAGE band-shift assay shows folding of the outer membrane protease OmpT in RL at a level fully comparable to that in DDM (data not shown). Furthermore *P. aeruginosa* express OprF, an OMP which is widely considered as an ortholog of OmpA due to sequence similarity and predicted protein fold [5,50]. In addition to RL, biosurfactants include other glycolipids such as sophorolipids, cellobioselipids and mannosylerythritol lipids. Sophorolipids are produced in both anionic and non-ionic form and preliminary SDS–PAGE band-shift studies show that OmpA can fold in mixtures of anionic and non-ionic SL as well as in micelles of the pure acidic form (data not shown). Thus biosurfactants are likely to support folding of outer membrane proteins on a more general level.

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